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Identification of CTX-M-Type Extended-Spectrum-β-Lactamase Genes Using Real-Time PCR and Pyrosequencing[▽]

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CTX-M extended-spectrum β-lactamases (ESBLs) are increasingly prevalent worldwide among *Escherichia coli* bacteria, mostly in community-acquired urinary tract infections. Finding a fast and reliable technique for identification of CTX-M enzymes is becoming a challenge for the microbiology laboratory. A fast real-time PCR amplification technique, using degenerated primers specific for all the bla_{CTX-M} alleles, coupled to real-time pyrosequencing was developed. The five CTX-M groups were unambiguously identified by pyrosequencing a 13-bp DNA region. Further sequencing of an additional 16-bp region allowed further division into subgroups. Phylogenetic trees constructed with the entire bla_{CTX-M} genes and with both pyrosequenced regions (29 bp) gave similar results, suggesting that this technique, termed the real-time detection and sequencing method, has a powerful discriminatory ability. This high-throughput technique has been evaluated by screening 48 ESBL-producing *E. coli* isolates recovered from the Bicêtre hospital (France) in 2004. Forty-four of these strains were CTX-M positive by real-time PCR detection and direct pyrosequencing of the PCR products, which identified CTX-M-15 as the main CTX-M-type β-lactamase. Pulsed-field gel electrophoresis analysis of these strains revealed that several clones, of which one CTX-M-15-positive clone was predominant (60%), were identified both in nosocomial and in community-acquired isolates. The combination of real-time PCR with pyrosequencing represents a powerful tool for epidemiological studies of CTX-M producers. This assay has the potential to be used in a diagnostic laboratory since up to 96 bacterial isolates may be screened in less than 3 h.

Extended-spectrum β-lactamases (ESBLs) of the CTX-M type have been reported increasingly in gram-negative rods, mostly in *Escherichia coli* (4, 5, 9, 13, 17, 24, 31, 42). The first CTX-M β-lactamase (CTX-M-1/MEN-1) was characterized in *E. coli* strains isolated from German and Italian patients (2, 3). More than 50 variants, sharing 71 to 98% amino acid sequence identities, have been described and are divided now into five phylogenetic groups based on amino acid sequence identity. They are the CTX-M-1 group (including 22 members), the CTX-M-2 group (including 10 members), the CTX-M-8 group (including 3 members), the CTX-M-9 group (including 18 members), and the CTX-M-25 group (with 4 members). The prevalences of these different groups are variable depending on the geographical localization (31).

β-Lactamases of the CTX-M groups are structurally related to the naturally produced β-lactamases of various *Kluyvera* species (8, 14, 29, 31, 32). The CTX-M enzymes usually have higher activities against cefotaxime than ceftazidime, but several enzymes, such as CTX-M-15 and CTX-M-19, also hydrolyze ceftazidime efficiently (15, 31, 33), which may complicate the phenotypic identification of CTX-Ms. The bla_{CTX-M} β-lactamase genes are mostly located on plasmids, but Toho-1-like β-lactamase genes have also been identified on chromosomes (4).

Apart from standard PCR and gene sequencing, molecular detection techniques for ESBLs in general have been used for a few applications most suited for the TEM- and SHV-type ESBLs that were mostly prevalent among nosocomial isolates and not community-acquired pathogens (1, 4, 6, 20, 23, 27, 28, 35, 41). Recent innovations in real-time PCR have simplified PCR considerably. Pyrosequencing is a genetic analysis method based on the detection of pyrophosphate that is released during the synthesis of DNA, thus allowing real-time sequence determination (36). Pyrosequencing that allows fast identification of short DNA sequences (up to 100 bp [36]) has been used already for detection of resistance-conferring genes in various bacteria (10, 12, 39, 44).

This study describes the first application of the LightCycler real-time PCR assay for detection of CTX-M variants, combined with real-time pyrosequencing for discrimination between the different bla_{CTX-M} genes in clinically relevant gram-negative species. By use of this combined technology, the clusters of CTX-M enzymes were accurately identified among a collection of 48 ESBL-positive *E. coli* clinical isolates. Further division within a given CTX-M cluster into pyrosequencing subgroups was also possible.

MATERIALS AND METHODS

Bacterial strains. The following well-characterized CTX-M producer strains were used as controls to develop and optimize the novel detection method: *Salmonella enterica* serovar Typhimurium NC ($bla_{CTX-M-1}$) (17), *E. coli* Jab ($bla_{CTX-M-2}$) (18), *Enterobacter aerogenes* Ker (bla_{CTXM-3}) (18), *Kluyvera georgiana* CUETM 4246-74 ($bla_{CTX-M-8}$) (32), *Enterobacter cloacae* Rms1 ($bla_{CTX-M-9}$) (gift from C. Deschamps), *E. coli* Mai ($bla_{CTX-M-14}$) (18), *E. coli* Urh ($bla_{CTXM-15}$) (18), *E. coli* Rms2 ($bla_{CTX-M-16}$) (Gift from C. Deschamps), *Klebsiella pneumoniae* ILT-3 ($bla_{CTX-M-19}$) (34), and *E. coli* ESBL530 ($bla_{CTXM-25}$) (22). Four *Klebsiella oxytoca* clinical isolates from the Bicêtre hospital with chromosomal β-lactamases related to CTX-M (e.g., KOXY/K1) were used as controls. *E. coli* DH10B was used as a CTX-M-negative control strain. Forty-eight ESBL-pro-

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TABLE 1. Amplification and pyrosequencing primers for the bla_{CTX-M}-like genes used in this study

Primer	Position	Comment(s)	Sequence (5′ to 3′)[a]
CTX-M-A1bio	208–227[b]	Forward amplification primer biotinylated	b-SCVATGTGCAGYACCAGTAA
CTX-M-A5	412–431[b]	Reverse amplification primer	TGRGMAATCARYTTRTTCAT
CTX-M-A6	629–649[b]	Forward amplification primer	TGGTRAYRTGGMTBAARGGCA
CTX-M-A8bio	782–803[b]	Reverse amplification primer biotinylated	b-TGGGTRAARTARGTSACCAGAA
S1	317–338[b]	Sequencing primer 1	TCVGYAATSGGRYTRTAGTTAA
S2	703–720[b]	Sequencing primer 2	GGBGATAARACCGGCAGC
CTX-M-A1	208–227[b]	PCR amplification primer	SCVATGTGCAGYACCAGTAA
CTX-M-A2′	712–731[b]	PCR amplification primer	CCRTARYCDCMGCTGCCGGT
Pre-CTX-M-3b	950–931[c]	PCR amplification primer	CCGTTTCCGCTATTACAAAC
ISEcp-Prom+	6218–6236[d]	PCR amplification primer	TGCTCTGTGGATAAC TTGC
IS903-Bint	7805–7785[d]	PCR amplification primer	GCTTTTTGACTTTCCACTCGC

[a] b-, biotinylated. M denotes A or C. W denotes A or T. R denotes A or G. Y denotes C or T. B denotes C, G, or T. S denotes C or G. V denotes G, A, or C.
[b] Compared to the sequence for CTX-M-3 (GenBank accession number Y10278) (11).
[c] Compared to the sequence for GenBank accession number X92506 (3).
[d] Compared to the sequence for GenBank accession number AF458080 (34).

ducing *E. coli* isolates were identified in clinical samples (one isolate per patient) during 2004 at the Bicêtre hospital (Le Kremlin-Bicêtre, France) (Table 1).

Antimicrobial agents and susceptibility testing. Routine antibiograms were determined by the disk diffusion method on Mueller-Hinton agar (Bio-Rad, Marnes-La-Coquette, France), and the susceptibility breakpoints were as recommended by the Clinical and Laboratory Standards Institute (6) and by the French Society for Microbiology guidelines (SFM) (http://www.sfm.asso.fr/nouv/general.php?pa=2). The presence of ESBLs was inferred by a synergy image, using the double-disk synergy test that was performed with cefotaxime or ceftazidime and ticarcillin-clavulanic acid disks (26).

Nucleic acid extractions. Whole-cell DNAs were extracted either from standard strains using a QIAamp DNA mini kit (QIAGEN, Les Ulis, France) or from clinical isolates and standard strains by a boiling extract procedure, using a few colonies of each bacterial strain resuspended in 100 μl of distilled water. After heating at 100°C for 10 min, freezing at −80°C for 10 min, and boiling for five additional min, the suspensions were centrifuged (5 min, 10,000 × *g*) and the recovered supernatant was frozen at −20°C until use.

PCR and Sanger DNA sequencing. PCR was performed on an ABI 2700 thermocycler, using laboratory-designed primers for detection and sequencing of the entire bla_{CTX-M}-like genes, as described previously (16, 18, 38) (Table 1). Two microliters of boiling extract supernatant was used as a template. PCR experiments were performed with 35 cycles consisting of 45 s of denaturation at 94°C, 45 s of annealing at 57°C, and 60 s of extension at 72°C. Both strands of the PCR products were sequenced with an Applied Biosystems sequencer (ABI 3100). The nucleotide and deduced amino acid sequences were analyzed and compared to sequences available over the Internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

Sequence alignment and phylogenetic tree construction. Multiple sequence alignments were performed online at the EBI bioinformatics website (http://www.ebi.ac.uk/clustalw/) using the program ClustalW, which calculates the best match for the selected sequences. The evolutionary relationships between sequences, based on the phylogenetic calculations of the neighbor-joining method of Saitou and Nei (37), have been displayed as phylograms, where the branch lengths are proportional to the amounts of inferred evolutionary change.

Real-time PCR. The oligonucleotide primers for PCR targeted two regions of CTX-M genes. The primers and probes were obtained from Sigma Proligo (Saint-Quentin Fallavier, France). The sequences for the primers are shown in Table 1 and Fig. 1. Primer pairs CTX-M-A1bio/CTX-M-A5 and CTX-M-A8bio/CTX-M-A6 have been designed in order to amplify two fragments of 224 bp and 175 bp, respectively, from each of the known bla_{CTX-M} genes (Table 1 and Fig. 1). The CTX-M-A1bio and CTX-M-A8bio primers were biotin labeled at their 5′ ends. All the primers and probes for real-time PCR and pyrosequencing were purified by high-performance liquid chromatography (Sigma Proligo).

PCR was performed using the LightCycler 2.0 system (Roche Diagnostics, Meylan, France) by rapid cycling in a reaction volume of 20 μl with amplification primers, each at a final concentration of 0.5 μM. LightCycler-FastStart DNA Master plus SYBR green I (Roche) was used. To complete the PCR mixtures, 15 μl of the master mixture and 5 μl of a DNA preparation were loaded into glass capillary cuvettes (Roche). After a short centrifugation (3,000 × *g* for 10 s), the sealed capillaries were placed into the LightCycler rotor.

The reaction protocol was as follows: an initial FastStart *Taq* DNA polymerase activation phase at 95°C for 10 min; a 45-cycle amplification phase consisting of a 95°C denaturation segment for 10 s, a 57°C annealing segment for 10 s, and a 72°C extension segment for 10 s; a melting phase from 55 to 95°C with a temperature transition rate of 0.1°C/s; and a rapid cooling phase. The quantity of the amplified product was monitored by detection of fluorescence energy emitted at 530 nm by SYBR green.

Melting curve analysis. The LightCycler system combines real-time PCR with a postamplification melting point analysis. The ability of the LightCycler system to perform melting curve analyses also allowed confirmation of the specificity of the amplified product and allowed for differentiation of CTX-M groups. The melting curve for each specimen was analyzed manually to determine the melting temperature (T_m). The T_m is the peak of the curve of the derivative of fluorescence (dF) with respect to temperature (dF/dT). By using the manual T_m function of the LightCycler software, the T_m was defined as the temperature at which the cursor just covered the highest point on the melting curve (the dF/dT curve). If the highest point was a plateau, the midpoint of the plateau was taken to be the T_m.

Sample recovery and control analysis of the amplification product. In order to verify the sizes of selected amplification products and the numbers of products for both amplification primer pairs, the capillaries were opened after the run in the LightCycler instrument and placed upside down in Eppendorf tubes. After a brief centrifugation, 5 μl of each sample was analyzed by 1.5% agarose gel electrophoresis (38).

Real-time pyrophosphate sequencing. Pyrosequencing was performed by using a PSQ 96 sample preparation kit and a PSQ 96MA analyzer (AB Biotage, Uppsala, Sweden), following the manufacturer's instructions. Unpurified amplified products recovered after real-time PCR were captured and separated by using streptavidin-Sepharose beads, and the resulting single-stranded DNA was used as a template for pyrosequencing with appropriate pyrosequencing probes (Fig. 1). The overall experiment is based on a protocol detailed on the Pyrosequencing Inc. website. Briefly, in pyrosequencing, a sequencing primer is annealed to a single-stranded PCR product, and nucleotides are added to the reaction mixture. Incorporation of the nucleotide by DNA polymerase leads to the release of pyrophosphate, which is further processed by sulfurylase and luciferase, producing light in proportion to the amount of pyrophosphate. The amount of light released at each extension step is presented as a pyrogram, and the relative numbers of a given nucleotide are consequently reflected by the relative peak heights on data traces. Excess nucleotides are enzymatically degraded before the following nucleotide is added.

PFGE. Whole-cell DNA from bla_{CTX-M}-producing *E. coli* isolates was analyzed by pulsed-field gel electrophoresis (PFGE) using XbaI (Amersham Biosciences, Les Ulis, France) with a CHEF DRII apparatus (Bio-Rad) as previously described (25). XbaI macrorestriction patterns were digitized and analyzed using Taxotron software (Institut Pasteur, Paris, France) and interpreted according to Tenover et al. (40).

RESULTS

Real-time PCR detection of bla_{CTX-M} genes in control strains. Fluorescence monitoring of product accumulation and detection by SYBR green of the bla_{CTX-M} genes were achieved

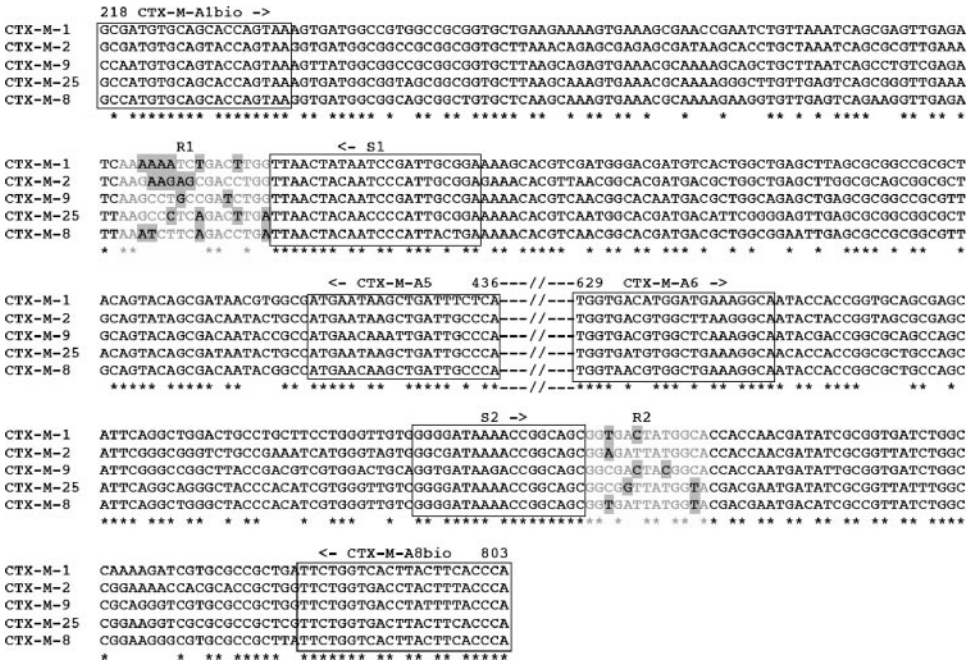

FIG. 1. Alignment of the nucleotide sequences of genes encoding β-lactamases CTXM-1, CTXM-2, CTXM-8, CTXM-9, and CTXM-25 based on the Lahey website (http://www.lahey.org/Studies/other.asp#table%201). PCR amplification primers (CTXM-A1bio, CTXM-A5, CTXM-A6, and CTXM-A8bio) and sequencing primers (S1 and S2) are indicated with open boxes, and the sense and antisense directions are indicated with arrows. Double slashes have been introduced into the sequences in order to show sequence interruptions. The sequenced regions are indicated in gray letters, and the polymorphic nucleotides in that region are highlighted. Nucleotide numbering of the $bla_{\text{CTX-M}}$ genes is indicated on the right-hand side of the nucleotide sequences.

during DNA amplification. The fluorescence signals rose above the background levels after about 20 cycles, when ca. 50 ng of QIAGEN kit-extracted whole-cell DNA was used as a template. No increase in the fluorescence signal was observed with the four *K. oxytoca* clinical isolates or with the negative controls, i.e., the *E. coli* DH10B isolate and the Tris-EDTA solution, during the whole run of 45 cycles (data not shown).

The melting curves for the amplification products with the primer pair CTX-M-A1bio and CTX-M-A-5 were systematically analyzed. The peaks obtained after mathematical transformation were 86.5°C for $bla_{\text{CTX-M-3/15}}$, 86.9°C for $bla_{\text{CTX-M-1}}$, 87.5°C for $bla_{\text{CTX-M-8}}$, 87.8°C for $bla_{\text{CTX-M-25}}$, 88.2°C for $bla_{\text{CTX-M-2}}$, and 88.5°C for $bla_{\text{CTX-M-9/14/16/19}}$. Even though very slight, these differences have been obtained reproducibly. Similar results were obtained with primer pair CTX-M-A6 and CTX-M-A8bio (data not shown). Additionally, monitoring by gel electrophoresis (data not shown) revealed one major amplification product of high intensity and expected length for each amplification primer pair (224 bp for CTX-M-A1bio/CTX-M-A5 and 175 bp for CTX-M-A6/CTX-M-A8bio). These products were absent from the *K. oxytoca* isolates, the *E. coli* DH10B isolate, and the Tris-EDTA negative control (data not shown).

Real-time sequencing. The high intensities of the PCR products obtained from each amplification primer pair were consistent with a use for pyrosequencing. The entire 20 μl of each PCR product was used for pyrosequencing template preparation, without initial purification. The amount of light released at each extension step is directly proportional to the amount of nucleotide added, the relative numbers of a given nucleotide being consequently reflected by the relative peak heights. Since the amplification product was of high intensity, the emitted light at each extension step was also of high intensity and clearly above the background level. In a few minutes, pyrosequencing distinguished among the genes encoding the five groups of CTX-M β-lactamases used as controls. The pyrograms obtained with three control CTX-M producer strains (CTX-M-1, CTX-M-3, and CTX-M-15) are illustrated in Fig. 2. Using the second sequencing primer, the S2 primer, differences in nucleotide sequences within a given group were obtained, especially within the CTX-M-1 group, allowing differentiation of the genes encoding CTX-M-1-, CTX-M-3-, and CTX-M-15-type enzymes (Fig. 2).

In order to evaluate the discriminatory power of pyrosequencing, the 29 pyrosequenced nucleotides from each of the known $bla_{\text{CTX-M}}$ genes have been aligned (Fig. 3). It is obvious from this alignment that pyrosequencing may rapidly distinguish between the five CTX-M groups but may also extend CTX-M differentiation within individual CTX-M groups. As an example, CTX-M group 1 may be further divided into eight pyrosequencing subgroups (named A to H). Each of these subgroups may contain one or several $bla_{\text{CTX-M}}$ genes. Pyrosequencing 29 bp has of course less discriminatory power than sequencing the entire gene. However, in order to compare both techniques, phylogenetic trees were constructed using either the two pyrosequenced regions, representing 29 nucleotides, or the entire gene for all the $bla_{\text{CTX-M}}$ gene sequences (Fig. 4) available at the Lahey Clinic (http://www.lahey.org/Studies) and/or the GenBank database (http://www.ncbi.nlm.nih.gov). The obtained phylogenetic trees were very similar

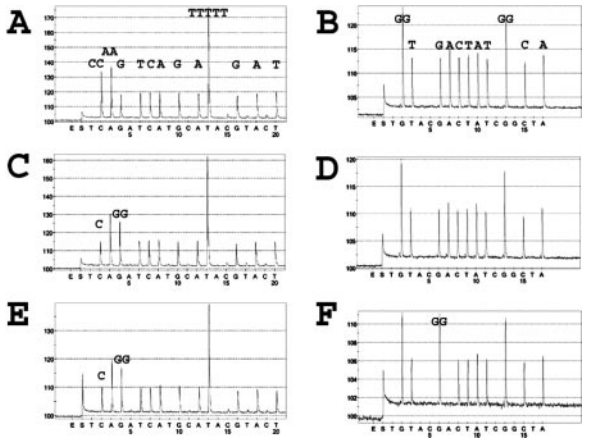

FIG. 2. (Left panels) Pyrosequencing of PCR products obtained with primers CTX-M-A1bio and CTX-M-A5 and DNAs extracted from standard strains $bla_{CTX\text{-}M\text{-}1}$ (A), $bla_{CTX\text{-}M\text{-}3}$ (C), and $bla_{CTX\text{-}M\text{-}15}$ (E), using the S1 sequencing primer. (Right panels) PCR products obtained with CTX-M-A6 and CTX-M-A8bio and DNAs extracted from standard strains $bla_{CTX\text{-}M\text{-}1}$ (B), $bla_{CTX\text{-}M\text{-}3}$ (D), and $bla_{CTX\text{-}M\text{-}15}$ (F), using the S2 sequencing primer. The scale on the left-hand side indicates the relative levels of fluorescence emitted during the pyrosequencing reaction. The corresponding nucleotide sequence is indicated above the obtained pyrogram for the $bla_{CTX\text{-}M\text{-}1}$ sequence (A and B). For the remaining programs, only nucleotides different from those in the $bla_{CTX\text{-}M\text{-}1}$ sequence are indicated.

CTX-M name	Genbank accession number	Pyrosequence with primer <-S1	S2->	CTX-M group (Pyrosubgroup)
FEC-1	AB098539	AAAAAATCTGACCTGG	GGTGACTATGGCA	1(A)
CTX-M-12	AF305837	AAAAAATCTGACCTGG	GGTGACTATGGCA	
CTX-M-30	AY292654	AAAAAATCTGACCTGG	GGTGACTATGGCA	
CTX-M-29	AY267213	AAAAAATCTGACCTGG	GGTGGCTATGGCA	1(B)
CTX-M-15	AY044436	AAAAAATCTGACCTTG	GGTGGCTATGGCA	1(C)
UOE-1	AY013478	AAAAAATCTGACCTTG	GGTGGCTATGGCA	
CTX-M-33	AY238472	AAAAAATCTGACCTTG	GGTGGCTATGGCA	
CTX-M-11	AY005110	AAAAAATCTGACCTTG	GGTGGCTATGGCA	
CTX-M-28	AJ549244	AAAAAATCTGACCTTG	GGTGGCTATGGCA	
CTX-M-3	Y10278	AAAAAATCTGACCTTG	GGTGACTATGGCA	1(D)
CTX-M-44[a]	DQ303459	AAAAAATCTGACCTTG	GGTGACTATGGCA	
CTX-M-42	AAY84742	AAAAAATCTGACCTTG	GGTGACTATGGCA	
CTX-M-52	DQ223685	AAAAAATCTGACCTTG	GGTGACTATGGCA	
CTX-M-22	AY080894	AAAAAATCTGACCTTG	GGTGACTATGGCA	
CTX-M-32	AJ557142	AAAAAATCTGACCTTG	GGTGACTATGGCA	
CTX-M-1	X92506	AAAAAATCTGACTTGG	GGTGACTATGGCA	1(E)
CTX-M-36	AB177384	AAAAAATCTGACTTGG	GGTGACTATGGCA	
CTX-M-23	AF488377	AAAAAATCTGACTTGG	GGTGACTATGGCA	
CTX-M-10	AF255298	AAAAAATCTGACCTGG	GGTGACTATGGTA	1(F)
CTX-M-37	AY649755	AAAAAATCTGACCTGG	GGTGACTATGGTA	
CTX-M-53	DQ268764	AAAAAATCTGACCTGG	GGTGGCTATGGTA	1(G)
CTX-M-34	AY515297	AAAAAATCTGACCTGG	TGTGACTATGGTA	1(H)
CTX-M-2	X92507	AAGAAGAGCGACCTGG	GGAGATTATGGCA	2(A)
CTX-M-31	AJ567481	AAGAAGAGCGACCTGG	GGAGATTATGGCA	
CTX-M-35	AB176532	AAGAAGAGCGACCTGG	GGAGATTATGGCA	
CTX-M-44	D37839	AAGAAGAGCGACCTGG	GGAGATTATGGCA	
CTX-M-20	AJ416344	AAGAAGAGCGACCTGG	GGAGATTATGGCA	
CTX-M-5	U95364	AAGAAGAGCGACCTGG	GGAGATTATGGCA	
CTX-M-43	DQ102702	AAGAAGAGCGACCTGG	GGAGGTTATGGCA	
CTX-M-4	Y14156	AGAGCAAGCGACCTGG	GGAGATTATGGCA	2(B)
CTX-M-7	AJ005045	AGAGCAAGCGACCTGG	GGAGATTATGGCA	
CTX-M-6	AJ005044	AAGGCGAGCGACCTGG	GGAGATTATGGCA	2(C)
CTX-M-16	AY029068	AAGCCTGCCGATCTGG	GGCGGCTACGGCA	9(A)
CTX-M-27	AY156923	AAGCCTGCCGATCTGG	GGCGGCTACGGCA	
CTX-M-9	AF174129	AAGCCTGCCGATCTGG	GGCGACTACGGCA	9(B)
CTX-M-51	DQ211987	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-14	AF252622	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-24	AY143430	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-18	AF325133	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-38	AY822595	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-19	AF325134	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-45	D89862	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-46	AY847147	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-50	AY847146	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-48	AY847144	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-47	AY847143	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-49	AY847145	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-13	AF252623	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-17	AY033516	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-21	AJ416346	AAGCCTGCCGATCTGG	GGCGACTACGGCA	
CTX-M-40	AY750914	AAATCCTCGGACCTGA	GGTGATTATGGTA	8(A)
CTX-M-41[a]	BAD90119	AAATCCTCGGACCTGA	GGTGATTATGGTA	
CTX-M-8	AF189721	AAATCTTCAGACCTGA	GGTGATTATGGTA	8(B)
CTX-M-25	AF518567	AAGCCCTCAGACTTGA	GGCGGTTATGGTA	25(A)
CTX-M-26	AY157676	AAGCCCTCAGACTTGA	GGCGATTATGGTA	25(B)
CTX-M-39	AY954516	AAGCCCTCAGACTTGA	GGCGATTATGGTA	
CTX-M-41	AAY43008	AAGCCCTCAGACTTGG	GGCGGTTATGGTA	25(C)
		* ** *	* * ** ** *	

FIG. 3. Nucleotide sequence alignment of 57 $bla_{CTX\text{-}M}$ genes covering only the two pyrosequenced regions, S1 and S2. The orientations of the sequencing primers are indicated above the nucleotide sequences with arrows. Sequences whose GenBank accession numbers are different from the numbers assigned to them at the Lahey website (http://www.lahey.org/Studies/other.asp#table%201) are represented with a superscript "a". Boldfaced names represent sequences present in the GenBank database and absent from the Lahey website. Accession numbers are indicated. Polymorphic nucleotides are highlighted in gray. The corresponding pyrosequencing subgroups are indicated in the right-hand column.

and grouped the β-lactamases in the same general manner (Fig. 4), suggesting that by pyrosequencing as few as 29 nucleotides, high discrimination not only among the different groups but also within a given group can be achieved.

Screening of ESBL-producing *E. coli* clinical isolates. In 2004, out of 4,200 clinical samples containing *Enterobacteriaceae* at the Bicêtre hospital in France, 86 (2.1%) had an ESBL phenotype according to the results of the double-disk antibiotic susceptibility tests. Since *E. coli* was by far the most prevalent ESBL-producing species (56%) and since CTX-M-type enzymes are mostly found in that species (4, 31), our work focused exclusively on *E. coli*. These 48 ESBL-producing *E. coli* isolates, representing 2% of the *E. coli* isolates (one *E. coli* isolate per patient) identified from clinical samples taken in 2004, were recovered mostly from urine (86%) and were isolated in locations scattered throughout the entire hospital, most predominantly in the gerontology (40%) and the emergency (21%) units (Table 2).

LightCycler PCR revealed that 44 out the 48 ESBL-producing *E. coli* isolates harbored a $bla_{CTX\text{-}M}$ gene (Table 2). Using pyrosequencing, the CTX-M enzymes detected belonged to three of the five CTX-M clusters, i.e., the CTX-M-1, CTX-M-2, and CTX-M-9 clusters, and to five pyrosequencing subgroups, i.e., CTX-M-1(C) ($n = 37$), CTX-M-1(E) ($n = 1$), CTX-M-2(A) ($n = 1$), CTX-M-9(A) ($n = 1$), and CTX-M-9(B) ($n = 4$) (Table 2). Using the LightCycler technology, up to 30 isolates were screened in one run of 50 min; thus, two runs were necessary to screen 48 isolates with one primer pair, and two additional runs were necessary for the second primer pair. The 44 CTX-M-positive *E. coli* isolates were genotyped with both pyrosequencing primers in less than 1 hour (30 min for sample preparation and 20 min for the pyrosequencing run). Overall, it took about 3 h to analyze 44 ESBL-producing *E. coli* isolates with one primer pair and less than 4 h with both primer pairs; since pyrosequencing allows up to 96 sequencing reactions in a single run, both PCR products from the 44 isolates could be pyrosequenced simultaneously.

The pyrosequencing-determined DNA sequence matched the DNA sequence obtained with classical Sanger DNA sequencing (Table 2). Results obtained with classical PCR followed by Sanger sequencing showed CTX-M cluster distributions identical to those obtained using the CTX-M real-time detection and sequencing method (Table 2). Similarly, the five different types of CTX-M enzymes detected, i.e., CTX-M-15 ($n = 37$), CTX-M-1 ($n = 1$), CTX-M-2 ($n = 1$), CTX-M-27 ($n = 1$), and CTX-M-14 ($n = 4$), matched perfectly with the five defined pyrosequencing subgroups, being CTX-M-1(C) ($n = 37$),

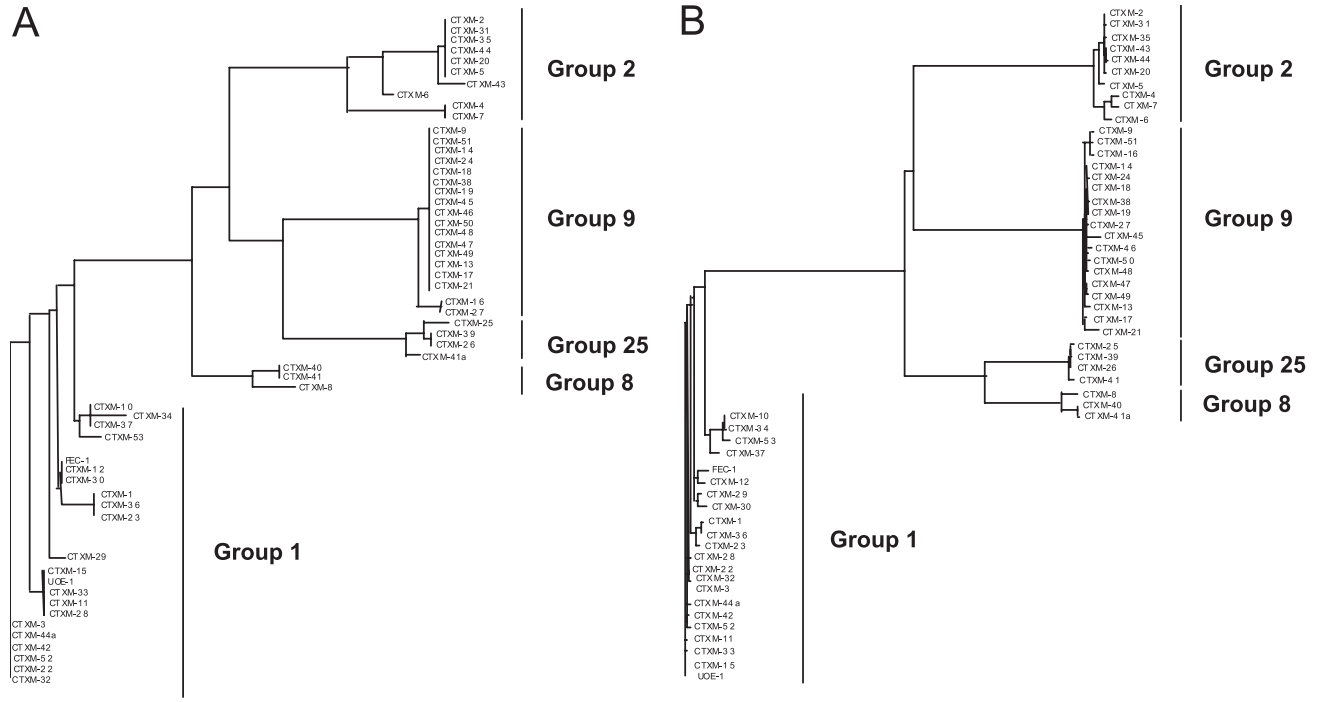

FIG. 4. Dendrogram obtained for 57 CTX-M enzymes by ClustalW analysis (37) of the two pyrosequenced regions, corresponding to 29 bp (A) and the entire *bla*$_{\text{CTX-M}}$ nucleotide sequence (B). Branch lengths are drawn to scale and are proportional to the numbers of changes.

CTX-M-1(E) (n = 1), CTX-M-2(A) (n = 1), CTX-M-9(A) (n = 1), and CTX-M-9(B) (n = 4), respectively (Table 2).

Epidemiology of ESBL-producing *E. coli* clinical isolates. Genotyping by PFGE revealed that 60% of CTX-M-producing *E. coli* isolates were clonally related. Whereas most isolates belonged to several subtypes of a given clone, differing by only one or two bands, several strains were unrelated (Fig. 5). While most of the CTX-M-15 isolates belonged to a single clone (Fig. 5) and were mostly found in the gerontology unit, where they seem to be epidemic, some clonally related isolates were found in the entire hospital and even in the emergency ward, where they were isolated from patients coming directly from the community.

DISCUSSION

Although ESBL production may lead to failure of treatment with expanded-spectrum cephalosporins, the MICs of expanded-spectrum cephalosporins for ESBL producers may be increased only slightly compared to those for non-ESBL producers (30). Consequently, clinical detection of ESBLs in clinical isolates is difficult (19, 30). Indeed, in a European survey, up to 33% of ESBLs in Europe may remain undetected (19). A series of easy-to-carry-out tests, mostly based on synergy between clavulanic acid and expanded-spectrum cephalosporins, are recommended, but their main pitfall is a limited sensitivity and the requirement of an overnight incubation. Detection of ESBLs at the genetic level represents an interesting alternative, which is independent of the degree of gene expression (7). A few methods that had been designed for TEM and SHV variants, such as oligotyping of *bla*$_{\text{TEM}}$ genes (20), single-strand conformational polymorphism analysis-PCR, and PCR-restriction fragment length polymorphism for *bla*$_{\text{SHV}}$ genes (23, 28), have now been overwhelmed by the diversity of types recognized since their first description. The disadvantages of the molecular-biology-based methods, such as labor expense, costliness, and the lack of general applicability, have outweighed their advantages and have so far prevented their broad acceptance. Real-time PCR coupled to melting curve analysis has been developed for several SHV- and GES-type ESBLs (35, 41). These techniques are interesting for investigating single-nucleotide mismatches within a sequence but are inappropriate for the study of a highly divergent family of β-lactamases, such as the CTX-Ms. Very recently, a multiplex PCR for detection of *bla*$_{\text{CTX-M}}$ genes was able to discriminate between the five CTX-M-clusters based on amplicon size analysis after agarose gel electrophoresis of the amplicons (43).

The method presented here is a rapid tool for identification of *bla*$_{\text{CTX-M}}$-type genes based on real sequence data, which is the gold standard for genetic information and thus the best assurance of a correct test. The PCR assay developed includes a first primer pair able to detect the known *bla*$_{\text{CTX-M}}$ genes emerging in various countries. Pyrosequencing performed as a complementary step allows the identification of the subgroup of the CTX enzyme in a few minutes. Pyrosequencing of the PCR-generated product using the second primer pair allows further enzyme differentiation. DNA obtained by boiling extraction of bacterial colonies from plates yielded high-intensity PCR products, consistent with a direct use for pyrosequencing, suggesting that accurate detection of *bla*$_{\text{CTX-M}}$ genes is possible regardless of the vastly various amounts of template DNA available from clinical isolates. Furthermore, the presence of minor amplification products in some amplification reactions

TABLE 2. Screening of bla_{CTX-M}-like genes in ESBL-producing *E. coli* clinical isolates from the Bicêtre hospital isolated in 2004

Strain no.	Date of isolation	Ward	Sample source	Status[a]	LC-PCR and pyrosequencing group[c]	Classical PCR and Sanger sequencing group
1	12 Jan. 2004	ICU[d]	Tracheal aspirate	C	CTX-M-1 (C)	CTX-M-15
2	27 Jan. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
3	07 Feb. 2004	Urology	Urine	N	CTX-M-9 (A)	CTX-M-27
4	12 Feb. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
5	16 Feb. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
6	17 Feb. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
7	17 Feb. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
8	05 Mar. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
9	11 Mar. 2004	Gastroenterology	Bile	N	CTX-M-1 (C)	CTX-M-15
10	18 Mar. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
11	19 Mar. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
12	21 Mar. 2004	Emergency	Urine	N	CTX-M-1 (C)	CTX-M-15
13	26 Mar. 2004	Emergency	Urine	N	CTX-M-1 (C)	CTX-M-15
14	05 Apr. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
15	05 Apr. 2004	Emergency	Urine	C	CTX-M-1 (C)	CTX-M-15
16	07 Apr. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
17	24 Apr. 2004	Nephrology	Urine	C	CTX-M-9 (B)	CTX-M-14
18	27 Apr. 2004	Neurology	Urine	N	CTX-M-9 (B)	CTX-M-14
19	04 May 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
20	25 May 2004	Emergency	Urine	C	CTX-M-1 (C)	CTX-M-15
21	25 May 2004	Urology	Urine	C	CTX-M-1 (C)	CTX-M-15
22	29 May 2004	Neurology	Urine	N	CTX-M-9 (B)	CTX-M-14
23	10 June 2004	Orthopedics	Urine	N	CTX-M-1 (C)	CTX-M-15
24	22 June 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
25	24 June 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
26[b]	16 July 2004	Internal medicine	Urine	N		
27[b]	21 July 2004	Emergency	Urine	C		
28	23 July 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
29	16 Sept. 2004	Neurology	Urine	N	CTX-M-1 (C)	CTX-M-15
30	16 Sept. 2004	Emergency	Urine	C	CTX-M-1 (C)	CTX-M-15
31	17 Sept. 2004	Emergency	Urine	N	CTX-M-1 (C)	CTX-M-15
32[b]	17 Sept. 2004	ICU	Urine	N		
33	29 Sept. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
34	11 Oct. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
35	13 Oct. 2004	Nephrology	Urine	C	CTX-M-2 (A)	CTX-M-2
36	15 Oct. 2004	Internal medicine	Tracheal aspirate	N	CTX-M-1 (C)	CTX-M-15
37	24 Oct. 2004	ICU	Catheter	N	CTX-M-1 (C)	CTX-M-15
38	22 Oct. 2004	Emergency	Urine		CTX-M-1 (C)	CTX-M-15
39	29 Oct. 2004	Gerontology	Urine	N	CTX-M-9 (B)	CTX-M-14
40	29 Oct. 2004	Emergency	Urine	C	CTX-M-1 (C)	CTX-M-15
41	04 Nov. 2004	Gastroenterology	Blood	C	CTX-M-1 (C)	CTX-M-15
42	16 Nov. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15
43	17 Nov. 2004	Orthopedics	Urine	C	CTX-M-1 (E)	CTX-M-1
44	23 Nov. 2004	Orthopedics	Urine	N	CTX-M-1 (C)	CTX-M-15
45	27 Nov. 2004	Emergency	Blood	C	CTX-M-1 (C)	CTX-M-15
46[b]	01 Dec. 2004	Pediatrics	Blood	N		
47	16 Dec. 2004	Neurology	Urine	C	CTX-M-1 (C)	CTX-M-15
48	29 Dec. 2004	Gerontology	Urine	N	CTX-M-1 (C)	CTX-M-15

[a] N, nosocomial infection; C, community-acquired infection.
[b] PCR results for these strains were CTX-M negative.
[c] Letters in parentheses indicate CTX-M subgroups. LC, LightCycler.
[d] ICU, intensive care unit.

did not interfere with the subsequent sequencing reaction (data not shown).

The rate of ESBL-producing enterobacterial isolates (2.1%) at the Bicêtre hospital has increased threefold since 2002, when it was only 0.7% (16). Similarly, the frequency of ESBL in *E. coli* has increased fivefold since 2002, when it was 0.38% (16). The CTX-M enzymes detected were of five different types, i.e., CTX-M-15 ($n = 37$), CTX-M-1 ($n = 1$), CTX-M-2 ($n = 1$), CTX-M-27 ($n = 1$), and CTX-M-14 ($n = 4$) (Table 2), belonging to three main CTX-M clusters, i.e., the CTX-M-1, CTX-M-2, and CTX-M-9 clusters. The $bla_{CTX-M-15}$ gene was predominant (37/44 CTX-M producers) in ESBL-producing *E. coli* isolates recovered from clinical samples at Bicêtre hospital, a situation that mirrors the current trend observed in other countries (4, 16, 31, 42). Again, the bla_{CTX-M} genes were always associated with insertion sequence IS*Ecp1*-like elements located upstream of the β-lactamase gene (data not shown), which are responsible for their expression and mobilization (33). In the present study, CTX-M enzymes represented 92% of the ESBLs produced by *E. coli* (44 of 48), which have increased significantly since 2002, when the frequency was only 60%, the other ESBLs being TEM derivatives (data not shown).

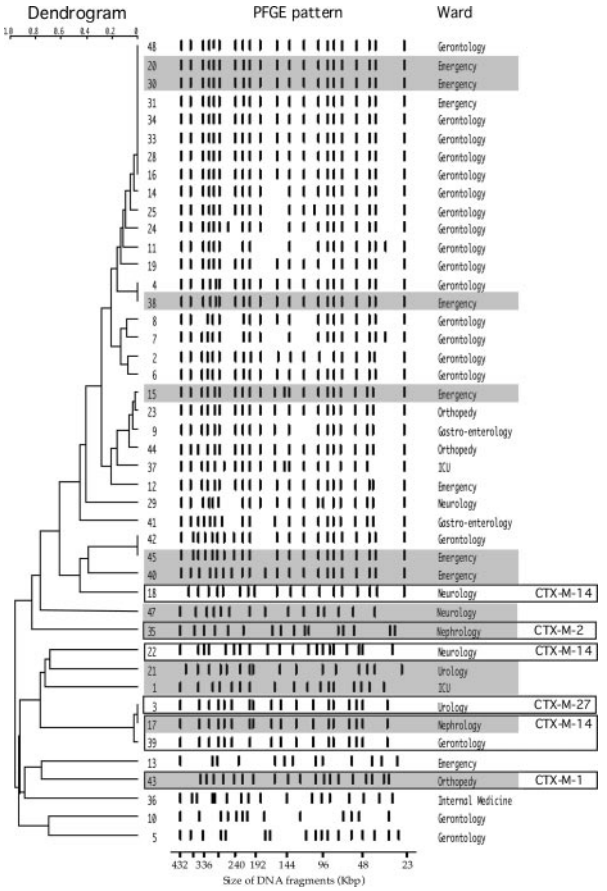

FIG. 5. Digitized PFGE patterns and phylogenetic tree of 44 CTX-M-producing *E. coli* isolates. XbaI macrorestriction patterns were digitized and analyzed using Taxotron software (Institut Pasteur, Paris, France) to calculate Dice coefficients of correlation and to generate a dendrogram by the unweighted pair group method using arithmetic averages. The scale is proportional to the level of pattern similarity. PFGE results were interpreted according to the criteria of Tenover et al. (40). The wards are indicated on the right.

The molecular epidemiology of the CTX-M-producing *E. coli* isolates in our institution was complex. Most CTX-M-15 isolates were epidemiologically related and were sufficiently similar to be considered subtypes of the same clone. One CTX-M-15 producer strain accounted for 60% of the isolates recovered from locations scattered throughout the hospital, most of which were isolated in the gerontology ward. Clonal diffusion of several pulsotypes was observed, likely the result of multiple importations of several unrelated clones from the community, as suggested from the observed diversity among strains from the Parisian area (9). The emergence of multiple CTX-M producers from the community, mostly observed here in the emergency unit, justifies by itself the rapid identification of CTX-M producers for subsequent isolation of strains from patients. Further development of this technique using clinical samples (such as urine) may help to further reduce the time for identification of CTX-M producers. Furthermore, multiplex PCR (both amplification primer pairs in one tube) followed by pyrosequencing may also reduce the overall time of the experiment to 3 hours. In addition, by coupling this technique with a rapid TEM and SHV PCR approach, most (if not all) of the currently spreading ESBLs in France may be identified.

In conclusion, ease, speed, and reliability render the real-time detection and sequencing method a powerful tool for epidemiological surveys concerning CTX-M producers and make it an interesting candidate for implementation into routine diagnostics. In geographical areas, such as in the United Kingdom, Spain, and France, where different CTX-M variants are known to coexist with a quite high prevalence (9, 13, 16, 21, 42), it might be useful to be able to distinguish these enzymes rapidly, avoiding the usual sequencing delays associated with classical methods. In addition, the pyrosequencing technique may also provide a rapid tool for determination of the molecular mechanisms of multidrug-resistant strains.

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